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Clinical detection of tumor progression is a major problem in treatment decisions for breast cancer patients. The ability to accurately detect hematogenous metastasis should significantly aid oncologist in disease management. To identify tumor progression more effectively than current approaches a less invasive, more accurate, quantitative assay is needed. Detection of metastatic tumor cells in blood and tissue by single marker Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) has shown potential value in clinical diagnosis. Major problems have been finding a reliable marker for this assays. Our laboratory has developed a multimarker RT-PCR assay that can reliably detect metastatic tumor cells in tissue and blood. We have successfully developed unique approach using multimarker, quantitative analysis and automated Southern blot detection system to provide highly stringent large scale analysis. Our experience in other cancers and viral nucleic detection will be applied to breast cancer. In this proposal we plan to test our hypothesis that a multimarker molecular assay can be clinically useful in detection hematogenous metastasis in breast cancer patients of different clinical and pathological stages of disease. The objective is to improve diagnosis and monitor patients for metastatic disease. A quantitative assay will be developed to determine the level of hematogenous metastasis and correlation to disease progression. To test our assay breast cancer patients of different clinical stages and, pre- and post-surgery operation blood will be monitored to determine the assays' efficiency and clinical relevance. The assay should provide significant improvement in breast cancer staging and management.

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ANNUAL REPORT

GRANT NO. DAMD17-96-1-6193
DAVE S.B.HOON, PHD

A QUANTITATIVE MULTIMARKER MOLECULAR ASSAY TO DETECT HEMATOGENOUS BREAST CANCER METASTASIS

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STUDY A: MULTIMARKER RT-PCR ASSAY TO DETECT OCCULT METASTATIC BREAST CANCER CELL

INTRODUCTION

Metastases to the axillary lymph nodes is the best predictor of patient outcome in breast cancer patients (Giuliano et al., 1994; Giuliano et al., 1995). Patients with lymph nodes free of metastases have a better outcome (Hellman et al., 1994; Quiet et al., 1995), but up to 30% will develop a relapse in 5-10 years (Hellman et al., 1994; Noguchi et al., 1996, Fisher et al., 1978). Serial sectioning and/or immunohistochemistry (IHC) of lymph nodes has increased detection of micrometastases by 10-30% compared to standard sectioning with hematoxylin and eosin (H&E) staining (Fisher et al., 1978; Ludwig Trial, 1990). The Ludwig trial demonstrated a poorer disease free and overall survival after 5 years median follow-up in patients with micrometastases detected on serial sectioning compared to patients which remained tumor free in the lymph node (Ludwig Trial, 1990). However, serial sectioning and IHC are labor intensive and requires examination of all lymph nodes to determine if micrometastatic disease is present. We previously demonstrated that the SN is the first node in the lymphatic basin to receive metastases from the primary tumor and is predictive of the pathology of the remaining lymphatic basin (Giuliano et al., 1995; Giuliano et al., 1997; Morton et al., 1992). In our experience, the SN can accurately predict axillary nodal status in at least 98% of cases (Giuliano et al., 1997). When we compared selective lymph node dissection (SLND) with standard axillary lymphadenectomy (ALND) for staging of breast cancer patients (Giuliano et al., 1995), the number of patients with axillary metastases (p < 0.03) and micrometastases (p < 0.0005) was significantly greater in the SLND group (Giuliano et al., 1995). This demonstrated that SLND, with multiple sectioning and immunohistochemical staining of the SN, increases the accuracy of axillary staging in breast cancer patients compared to ALND with routine histopathologic examination of lymph nodes. The SN, which predicts the pathology of the remaining basin, allows for an extensive and focused analysis of one or two nodes, rather than examination of all axillary nodes.

Recently, several investigators have reported using ultra sensitive molecular techniques to detect micrometastases in blood, bone marrow and lymph nodes by RT-PCR (Datta et al., 1994; Mori et al., 1995; Noguchi et al., 1996). This method, based on amplification of mRNA tumor markers expressed by breast cancer cells, has increased detection of occult metastases by 6 to 55% (Datta et al., 1994; Mori et al., 1995; Noguchi et al., 1996; Schoenfeld et al., 1994). Although tumors are heterogenous in gene expression, most studies have relied on a single tumor marker to detect micrometastases (Datta et al., 1994; Hoon et al., 1995a; Mori et al., 1995; Schoenfeld et al., 1994). Tumor cells within a primary tumor may all express the same marker gene, but the level of specific mRNA marker expression can vary. These biological tumor factors must be carefully considered in developing a sensitive, specific, and reliable RT-PCR detection assay. The use of multiple markers is one approach to improve the sensitivity and specificity of the RT-PCR assay system (Hoon et al., 1995b; Sarantou et al., 1997). This technique has not been previously applied to the SN in breast cancer patients.

We undertook this study to determine the expression of mRNA tumor markers C-Met, B1→4GalNAc-T, and P97 using RT-PCR and Southern blot assay in frozen section SN of breast cancer patients. The hepatocyte growth factor receptor (HGFR), also known as the product of the met proto-oncogene (C-Met), is expressed by a variety of epithelial (and a few non-epithelial) cell types including both benign and malignant mammary epithelium (Gherardi et al., 1991; Tsarfaty et al., 1992; Tuck et al., 1996). The binding of HGF to HGFR has been shown to enhance a variety of cell functions to include cell growth, movement, and invasiveness and clinically correlates with a shorter metastases-free and overall survival in breast cancer patients (Dean et al., 1985; Rahimi et al., 1994; Tuck et al., 1996). B1→4N-acetylgalactosaminyltransferase (B1→4GalNAc-T), is a carbohydrate transferase involved in the addition of GalNAc to gylcoproteins and glycosphingolipids (Pohlentz et al., 1988; Nagata et al., 1992; Yamashiro et al., 1993). The aberrant expression of a type of glycosphingolipid which requires B1→4GalNAc-T has been reported in renal, gastric, and breast carcinomas (Hoon et al., 1993; Brooks et al., 1995; Osinaga et al., 1994) Aberrant glycosylation of glycosphingolipids on tumor cells has been associated with specific events of tumor growth, invasion, and metastases (Hakormori et al., 1996). P97 (melanotransferrin), a cell surface glycoprotein, described as a marker antigen for human melanoma cells (Hoon et al., 1995b; Brown et al., 1981; Rose et al., 1986), is also expressed in breast, cervical, and thyroid cancers (Barresi and Tuccari. 1996). P97 has structural similarity to human transferrin and lactoferrin, but its function has not yet been determined.

MATERIAL AND METHODS

Breast cell lines

The breast cell lines MCF-7, BT-20, MDA-MB-231 were obtained from the ATCC (Rockville, MD) and cultured according to instructions. The 734B line is an established subclone of MCF-7. The established breast cancer cell lines JWCI BM-1 and JWCI JM-1 were developed from primary invasive ductal carcinoma and characterized as a breast cancer cell line at the John Wayne Cancer Institute. All 6 cell lines were grown in RPMI 1640 (Gemini Bioproducts, Calabasas, CA) plus 10% fetal calf serum (heat inactivated), penicillin and streptomycin (GIBCO, Grant Island, NY) in T75 cm^2 flask. Adherent cell lines were routinely passaged by trypsinization every 3-4 days (Hoon et al., 1989).

Surgical specimens and blood preparation

Surgical specimens were obtained in consultation with the surgeon and pathologist. All tissues were collected and dissected under stringent sterile conditions to prevent RNA contamination. Representative samples of primary breast malignant tissues and the above mentioned breast cancer cell lines were positive controls for establishing the multiple marker assay. Lymph nodes from patients undergoing surgery for noncancer (i.e. herniorraphy, cholecystectomy) conditions served as negative controls (internal review board approval for human subject usage was obtained).

The multiple marker RT-PCR assay was used to assess the tumor status of 57 SN from 41 AJCC stage I-IIIA breast cancer patients. Each SN was bisected; six 12ug frozen section slices were obtained from each half of the SN. These were immediately stored at -80^0 C until used for further examination by RT-PCR plus Southern blot assay. The remainder of the SN was examined with conventional H&E staining and by IHC

analysis when no tumor was detected by H&E. Cytokeratin IHC was performed using an antibody cocktail (MAK-6; Ciba-Corning, Alameda, CA) to low- and intermediate-molecular weight cytokeratins with an automated immunoperoxidase system (Ventana ES; Ventana Medical Systems, Inc., Tucson, AZ.).

Ten milliliters of blood were collected in sodium citrate-containing tubes, as previously described (Hoon et al., 1995b). The blood was centrifuged using a hypotonic density gradient solution (National Genetics Institute, Los Angeles, CA) and nucleated cells in the blood were collected for RNA isolation as previously described (Sarantou et al., 1997). Blood (PBL) from healthy donor volunteers served as negative controls.

TRI-REAGENT (Molecular Research Center, Cincinnati, OH) was used to isolate total RNA from the cell lines and frozen section SN, following the manufacturer's instructions. The sample was stored at -4^0 C for 12 hr. after adding 500 ul of isopropranol for precipitation. The tube was then centrifuged at 14,000 g at 4^0 C for 10 min. The sample was washed with 75% ethanol, vacuum-dried and suspended in 10mM Tris-HCl with 0.1 EDTA solution (pH 8). The concentration, purity, and amount of total RNA were determined by UV spectrophotometry. The integrity of all RNA samples were determined by performing RT-PCR for B-actin mRNA expression, and assessment by ethidium bromide gel electrophoresis. Tissue processing, RNA extraction, RT-PCR assay set-up and post-PCR product analysis were carried out in separate designated rooms in separate facilities to prevent cross-contamination as previously reported.

RT-PCR and Southern blot assay

The RT-PCR assay was carried out as previously described (Hoon et al., 1995b; Sarantou et al., 1997). Reverse transcription (RT) was performed on the amount of total RNA specified for Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). The RNA was incubated at 70^0 C for 5 min and then put on ice before addition of RT reaction reagents. The RNA with RT reagents added was incubated at 37^0 C for 2 hrs, followed by heating at 95^0 C for 5 min. All RT reactions were carried out with oligo dT priming to avoid transcription of spurious non-polyadenylated mRNA. The PCR conditions for B1→4GalNAC-T and P97 were set up as follows: 1 cycle of denaturing at 95^0 C for 5 min followed by 35 cycles of 95^0 C for 1 min, 65^0 C for 1 min, and 72^0 C for 1 min before a final primer sequence extension incubation at 72^0 C for 10 min. The PCR conditions for C-Met were similar, except an annealing temperature of 60^0 C was used. RT-PCR conditions were set up in an Omni thermocycler (Hybaid, Middlesex, UK). Oligonucleotide primers were synthesized and purified at National Genetics Institute. Optimal primer sequences were designed for specific C-Met, B1→4GalNAc-T, and P97 mRNA detection and cDNA products. Oligonucleotide 5' and 3' primers for individual markers were synthesized as follows: C-Met primers, 5'-GTC CTT TGG CGT CGT CCT CT-3' and 5'ATG GGC GCA TTT CGG CTT TA-3'; B1→4GalNAc-T, 5'CAA ACT CAA CAG GCA ACT AC-3' and 5'-GAT CAT AAC GGA GGA AGG TC-3' ; P-97, 5'-TAC CTG GTG GAG AGC GGC CGC CTC-3' and 5'AGC GTC TTC CCA TCC GTG T -3'. The RT-PCR cDNA products of C-Met, B1→4GalNAc-T, and P97 were 185, 230, and 286 base pairs, respectively. B-actin primers have been previously described (Hoon et al.; 1995b). A purified fragment of the cDNA products amplified with respective primer sets was labeled with digoxigenin

(Boehringer Mannheim, La Jolla, CA) and used as a cDNA probe in the automated Southern blot assay (National Genetics Institute) (Sarantou et al., 1997).

The automated Southern blot assay system provides RT-PCR product verification, with the highest stringency in blotting and sensitivity. Any discrepancies or faint signals in Southern blotting were repeated for verification. In each experiment set-up, samples of RT-PCR reagents without mRNA, donor PBL, and lymph node RNA from non-cancer patients were used as negative controls throughout RT-PCR procedures. Complementary DNA (cDNA) template for the cDNA probe and previously tested positive tumor lines or tumor biopsy specimens were included in the assay as positive RT-PCR controls. Southern blots were scanned on an electronic imager, recorded using computer software, and verified by at least two readers for positive and negative results. If known reagent control samples were different than expected, the results were discarded and the assay repeated with new reagents. A positive result was recorded only after verification by Southern blotting. This highly stringent system with multiple controls was designed to perform routine analysis of large number of samples while maintaining high specificity and consistency.

RESULTS

Marker expression in breast cell lines and breast tumors

All 6 breast cancer cell lines assessed expressed C-Met, B1→4GalNAc-T, and P97 (Table 1). A positive result was indicated by an 185 base pair (bp) cDNA band as detected by EtBr for C-Met, 230 bp for B1→4GalNAc-T, and 286 bp for P97. Southern blot analysis was used to verify the sensitivity and specificity of the assay (Fig. 1). Biopsies from ten primary infiltrating ductal carcinomas (IDC) were evaluated for mRNA tumor marker expression. B1→4GalNAc was expressed in 6 of 10 (60%) primary breast tumors and C-Met and P97 were expressed in 7 of 10 (70%).

Marker expression in normal PBL and lymph nodes

TABLE 1
RT-PCR & SOUTHERN BLOT ANALYSIS OF MRNA MARKER EXPRESION

SPECIMEN MRNA MARKER EXPRESSION

	C-MET	B1→4GALNAC-T	P97
BREAST CELL LINES (n=6)	6 (100)	6 (100)	6 (100)
BREAST TUMOR TISSUE (n=10)	7 (70)	6 (60)	7 (70)
HEALTHY DONOR PBL (n=25)	0	0	0
NORMAL LYMPH NODES (n=10)	1 (10)	0	1 (10)
SENTINEL LYMPH NODES (n=57)	43 (75)	34 (60)	36 (63)

RT-PCR marker expression refers to RT-PCR and Southern blot positive. All specimens assessed were *B*-actin RT-PCR positive by EtBr analysis. ( ) refers to % positive.

PBL from 25 volunteer healthy donors were evaluated for mRNA marker expression. None of the markers were expressed in these 25 healthy donor PBL under the optimal conditions described. The RT-PCR results were confirmed by EtBr and Southern blot assay.

After informed consent, ten normal lymph nodes (confirmed histopathologically), were obtained from patients undergoing non-cancer surgery (cholecystectomy, herniorraphy). No mRNA tumor markers were expressed in these normal lymph nodes by EtBr, but C-Met and P97 were expressed in 1 of 10 normal lymph nodes using Southern blot analysis. B1→4GalNAc-T was not expressed in normal lymph nodes by EtBr or Southern blot assay.

mRNA marker detection sensitivity

Total RNA was isolated from breast carcinoma cell lines and quantitated to determine the sensitivity of the assay. The total RNA was then serially diluted and RT-PCR and Southern blot was performed. RT-PCR was performed on serial dilution of RNA isolated from a representative breast cancer cell line, MCF-7. Detection of RT-PCR cDNA product could be detected by Southern blot analysis in 10^{-5} ug for C-Met and B1→4GalNAc-T, and 10^{-4} ug for P97. Most important is the amount of specific mRNA copy that can be detected.

Marker expression in frozen section sentinel nodes

Fifty-seven SN from 41 AJCC stage I-IIIA breast cancer patients undergoing selective lymph node dissection (SLND) were analyzed by RT-PCR and Southern blot assay for C-Met, B1→4GalNAc-T, and P97 mRNA expression (Table 2). Seventeen of 57 SN (30%) contained metastases; 10 (18%) detected with H&E and an additional 7 (12%) with IHC. The remaining 40 of 57 (70%) SN did not contain detectable metastases. C-Met mRNA was expressed in 43 of 57 (75 %) SN, B1→4 GalNAc-T mRNA in 34 of 57 (60%), and P97 mRNA in 36 of 57 (63%).

TABLE 2
COMPARISON OF HISTOPATHOLOGY VERSUS MRNA EXPRESSION OF SN

RT-PCR & SOUTHERN BLOT ANALYSIS OF mRNA MARKERS	PATHOLOGY OF SN BY H&E OR IHC	
	POSITIVE (%)	NEGATIVE (%)
C-MET		
POSITIVE	12 (71)	31 (78)
* NEGATIVE	5 (29)	9 (22)
B1→4GalNAc-T		
POSITIVE	13 (76)	21 (53)
NEGATIVE	4 (24)	19 (47)
P97		
POSITIVE	11 (65)	25 (63)
NEGATIVE	6 (35)	15 (37)

RT-PCR positive refers to RT-PCR and Southern blot positive. All specimens assessed were *B*-actin RT-PCR positive by EtBr analysis.

Marker expression in sentinel nodes tumor-free by H&E and IHC

In 40 SN (Table 2) without metastases by conventional H&E or IHC staining, 31 of 40 (78%) expressed C-Met, 21 of 40 (53%) expressed B1→4GalNAc, and 25 of 40 (63%) expressed P97. 5 of 40 SN (13%) expressed 0 markers (Table 3), 10 of 40 (25%) expressed 1 marker, 8 of 40 (20%) expressed 2 markers and 17 of 40 (42%) expressed all three mRNA markers. Twenty-five of 40 (62%) histopathology tumor-free SN expressed 2 or more markers.

TABLE 3
SENTINEL NODE RT-PCR & SOUTHERN BLOT MARKER
EXPRESSION RT-PCR AND SOUTHERN BLOT ANALYSIS

	NUMBER OF MARKERS				
SENTINEL NODES (n=57)	0(+)	1(+)	2(+)	3(+)	TOTAL
PATHOLOGY POSITIVE (n=17)	1	2	8	6	17
PATHOLOGY NEGATIVE (n=40)	5	10	8	17	40

Pathology positive refers to H&E staining or IHC staining positive specimens.

Marker expression in sentinel nodes with tumor by H&E or IHC

In 17 SN that contained metastases by conventional H&E staining or IHC, 12 of 17 (71 %) expressed C-Met, 13 of 17 (76%) expressed B1→4GalNAc-T, and 11of 17 (65 %) expressed P97. No individual tumor marker alone was expressed in all 17 SN. One of 17 (6%) SN expressed 0 markers, 2 of 17 (12 %) expressed 1 marker, 8 of 17 (47%) expressed 2 markers, and 6 of 17 (35%) expressed all three markers. Fourteen of 17 (82%) SN expressed 2 or more markers.

Heterogeneity of marker expression in patients with multiple sentinel nodes

Eleven (27 %) patients had more than one SN removed from their axillary basin (Table 4). In seven patients (64%), mRNA marker expression was the same (homogenous) in the multiple sentinel nodes (Patients 1-7), while in 4 (36%) marker expression varied (heterozygous) (Patients 8-11). In 3 of these 4 patients, the histopathology varied between the multiple sentinel nodes, which could explain the difference in mRNA marker expression.

TABLE 4

MARKER EXPRESSION IN PATIENTS WITH MULTIPLE SENTINEL NODES				
	H&E/IHC	C-Met	B1→4GalNAc-T	P97
Patient 1				
SN(1)	-	+	+	+
SN(2)	-	+	+	+
Patient 2				
SN(1)	+	+	-	-
SN(2)	+	+	-	-
Patient 3				
SN(1)	-	+	+ .	+
SN(2)	-	+	+	+
Patient 4				
SN(1)	-	+	+	+
SN(2)	-	+	+	+
Patient 5				
SN(1)	-	+	-	-
SN(2)	-	+	-	-
Patient 6				
SN(1)	-	+	+	+
SN(2)	-	+	+	+
Patient 7				
SN(1)	-	-	+	+
SN(2)	-	-	+	+
SN(3)	-	+	+	+
Patient 8				
SN(1)	-	-	-	-
SN(2)	+	-	+	+
Patient 9				
SN(1)	+	+	+	-
SN(2)	-	+	-	-
SN(3)	-	+	-	-
Patient 10				
SN(1)	+	-	+	+
SN(2)	+	-	+	+
SN(3)	-	+	-	+
Patient 11				
SN(1)	+	+	-	+
SN(2)	+	+	-	-

RT-PCR positive refers to RT-PCR and Southern blot positive. All specimens assessed were B-actin RT-PCR positive by EtBr analysis.

FIGURE 1 LEGEND

Representative examples of multiple marker RT-PCR and Southern blot assay of breast cancer patients. Lane 1 corresponds to RT-PCR-negative control (water); lane 2, healthy donor blood; lane 3, normal lymph node; lane 4, breast cancer cell line; lane 5, primary breast tumor (infiltrating ductal carcinoma); lane 6 and 7, SN; lane 8, positive marker Southern blot standard control.

CONCLUSIONS

In this study, we demonstrated the feasibility of performing RT-PCR and Southern blot analysis on frozen sections of SN using a multiple marker assay. The SN, which is predictive of the histopathology of the axillary basin, allows a more focused analysis of one or two nodes in staging breast cancer patients (Giuliano et al., 1997; Turner et al., 1997). We have combined the techniques of serial sectioning, immunohistochemistry, and RT-PCR with Southern blot analysis to increase the detection of micrometastases in the SN (Giuliano et al., 1994; Noguchi et al., 1996; Rosen et al., 1981). Designing a sensitive RT-PCR assay to detect occult metastases in lymph nodes of breast cancer patients has been limited by the number of mRNA tumor markers consistently present in tumor and not in normal lymph nodes (Noguchi et al., 1996; Mori et al., 1995; Hoon et al., 1995a). The ideal mRNA tumor marker, expressed at high levels in breast cancer cells and not at all in normal lymph nodes, has yet to be identified. Current mRNA markers used in RT-PCR analysis of breast cancer axillary nodes include MUC-1, cytokeratin 18 and 19, and CEA (Schoenfeld et al., 1994; Mori et al., 1995; Hoon et al., 1995a). We previously demonstrated that MUC1 mRNA was expressed in four of eight normal lymph nodes by RT-PCR and Southern blot analysis (Hoon et al., 1995a).

Patients with SN expressing more than one marker by RT-PCR may be more specific and accurate in predicting relapse compared to those expressing a single marker. We have previously shown in melanoma that patients expressing multiple markers in the blood had an earlier disease progression compared to those expressing a single marker (Hoon et al., 1995b). In this study, 25 of 40 (62%) histopathology tumor-free SN expressed 2 or more mRNA markers compared to 14 of 17 (82%) SN with metastases . One normal lymph node in this study expressed 2 markers (C-Met and P97), while the remaining lymph nodes did not express any. Long term follow-up is required to determine if patients that express multiple compared to single mRNA marker are more specific in predicting which patients are at risk of relapse.

No single marker alone was expressed in all seventeen SN that contained metastases by H&E or IHC. Failure of mRNA tumor markers to be expressed in all SN containing metastases by H&E or IHC may be due to sampling error (Noguchi et al., 1996; Hoon et al., 1996). In some cases only a small cluster of tumor cells in the subcapsular sinus were detected by IHC. Since a portion of the SN must always be preserved for conventional analysis, a sampling error can occur, especially in cases were tumor burden is minimal (Hoon et al., 1995b). Failure of mRNA marker expression by RT-PCR in SN containing metastases by H&E or IHC may also occur because of heterogeneity of tumor marker expression as demonstrated in this study. A tumor cell may express a specific marker gene at different stages of disease or the level of expression may be too low to be detected by the RT-PCR assay (Noguchi et al., 1996; Hoon et al., 1995b). For reasons described, a multiple marker, in comparison to a single marker, may improve the overall sensitivity and specificity of the RT-PCR assay. In this study, only one of 17 (6%) sentinel nodes with metastases by H&E or IHC did not express at least one of three mRNA tumor markers.

In conclusion, the multiple marker RT-PCR and Southern blot assay improves the detection of occult metastases in the SN when compared to conventional H&E and IHC analysis. Because of the heterogeneity of tumor marker

expression, the multiple marker compared to a single marker improves the overall sensitivity and specificity of the assay. The SN, which is predictive of the histopathology of the remaining axillary nodes allows a more focused and nonrandom analysis in staging breast cancer patients. Further clinical follow-up will help to determine whether patients who express mRNA tumor markers in the SN by RT-PCR and Southern blot analysis are at risk of relapse.

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SUDY B: ASSESSMENT OF NEW RT-PCR MARKERS FOR DETECTION OF METASTATIC BREAST CANCER CELLS

INTRODUCTION

The axillary lymph node status remains the most powerful predictor of outcome in breast cancer patients (Giuliano et al, 1994, 1995). However, the recurrence rates in patients that are histopathology tumor free in the axillary lymph nodes are as high as 30 to 50 % over 5 to 10 years (Hellman, 1994; McGuire and Clark, 1992). Prognostic factors to separate patients into low and high-risk groups in terms of probability of recurrence, death and need for adjuvant therapy has not been established (McGuire and Clark, 1992).

Improving the detection of micrometastases in the axillary lymph nodes is another approach to identify patients at risk of relapse. Serial sectioning and/or immunohistochemistry (IHC) staining has increased the detection of occult metastases from 9% to 33% (Ludwig International trial, 1990). In the Ludwig trial, patients who demonstrated micrometastases on serial sectioning had a poorer disease-free (p=0.003) and overall survival (p=0.002) after 5 years' median follow-up than patients whose nodes remained negative on serial sectioning. IHC and serial-sectioning of all axillary lymph nodes are both time-consuming and expensive, and has limited its use in clinical practice.

More recently RT-PCR has been developed as an ultra-sensitive method to detect metastases in the axillary lymph nodes. The reported increased detection of occult metastases by RT-PCR has ranged from 6% to 55% using CEA, Cytokeratin-19, and MUC-1 as mRNA markers (Mori et al, 1995; Schoenfeld et al, 1994; Noguchi et al, 1996). However, there is concern that non-cancer cells may express these mRNA markers and limit the specificity of the RT-PCR assay (Traweek et al, 1993; Hoon et al, 1995). Traweek et al (1993) has shown cytokeratin-8 and -18 expression in peripheral blood mononuclear cells, lymph nodes and bone marrow from non-cancer patients. Hoon et al (1995) demonstrated MUC-1 expression by RT-PCR in 4 of 8 lymph nodes from patients without cancer.

This study was undertaken to determine the sensitivity and specificity of RT-PCR and Southern blot assay in the lymph nodes from breast cancer patients using mRNA tumor markers Cytokeratin-19, Cytokeratin-20, CEA, MUC-1, and GA733.2. Limitations of RT-PCR in the axillary lymph nodes and the feasibility of a single mRNA marker in breast cancer patients are discussed.

MATERIAL AND METHODS

Breast cell lines

The breast cell lines MCF-7, BT-20, MDA-MB-231 were obtained from the ATCC (Rockville, MD) and cultured according to instructions. The 734B line is an established subclone of MCF-7. All 4 cell lines were grown in RPMI 1640 (Gemini Bioproducts, Calabasas, CA) plus 10% fetal calf serum (heat inactivated), penicillin and streptomycin (GIBCO, Grant Island, NY) in T75 cm^2 flask. Adherent cell lines were routinely passaged by trypsinization every 3-4 days (Hoon et al, 1989).

Surgical specimens and blood preparation

Surgical specimens were obtained in consultation with the surgeon and pathologist. All tissues were collected and dissected under stringent sterile conditions to prevent RNA contamination. Representative samples of primary breast malignant tissues and the above mentioned breast cancer cell lines were positive controls for the assay. Internal review board approval was obtained for human subject usage of lymph nodes from patients undergoing surgery for noncancer (i.e.herniorraphy, cholecystectomy) and served as negative controls.

The RT-PCR assay was used to assess the tumor status of SN from AJCC stage I-IIIA breast cancer patients. Each SN was bisected; six 12ug frozen section slices were obtained from each half of the SN. These were immediately stored at -80^{0}C until used for further examination by RT-PCR plus Southern blot assay. The remainder of the SN was examined with conventional H&E staining and by IHC analysis when no tumor was detected by H&E. Cytokeratin IHC was performed using an antibody cocktail (MAK-6; Ciba-Corning, Alameda, CA) to low- and intermediate-molecular weight cytokeratins with an automated immunoperoxidase system (Ventana ES; Ventana Medical Systems, Inc., Tucson, AZ.).
Ten milliliters of blood were collected in sodium citrate-containing tubes, as previously described (Hoon et al, 1995), from AJCC stage I-IIIA breast cancer patients and from healthy donor volunteers, which served as negative controls. The blood was centrifuged using a hypotonic density gradient solution (National Genetics Institute, Los Angeles,

CA) and nucleated cells in the blood were collected for RNA isolation as previously described (Sarantou et al, 1997).   TRI-REAGENT (Molecular Research Center, Cincinnati, OH) was used to isolate total RNA from the cell lines and frozen section SN, following the manufacturer's instructions. The sample was stored at -4^0 C for 12 hr. after adding 500 ul of isopropranol for precipitation. The tube was then centrifuged at 14,000 g at 4^0 C for 10 min. The sample was washed with 75% ethanol, vacuum-dried and suspended in 10mM Tris-HCl with 0.1 EDTA solution (pH 8). The concentration, purity, and amount of total RNA were determined by UV spectrophotometry. The integrity of all RNA samples were determined by performing RT-PCR for B-actin mRNA expression, and assessment by ethidium bromide gel electrophoresis. Tissue processing, RNA extraction, RT-PCR assay set-up and post-PCR product analysis were carried out in separate designated rooms in separate facilities to prevent cross-contamination as previously reported.

RT-PCR and Southern blot assay

The RT-PCR assay was carried out as previously described (Sarantou et al, 1997). Reverse transcription (RT) was performed on the amount of total RNA specified for Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). The RNA was incubated at 70^0 C for 5 min and then put on ice before addition of RT reaction reagents. The RNA with RT reagents added was incubated at 37^{0}C for 2 hrs, followed by heating at 95^0 C for 5 min. All RT reactions were carried out with oligodT priming to avoid transcription of spurious non-polyadenylated mRNA. RT-PCR conditions were set up in an Omni thermocycler (Hybaid, Middlesex, UK). Oligonucleotide primers were synthesized and purified at National Genetics Institute. B-actin primers have been previously described (Hoon et al, 1995). A purified fragment of the cDNA products amplified with respective primer sets were labeled with digoxigenin (Boehringer Mannheim, La Jolla, CA) and used as a cDNA probe in the automated Southern blot assay (National Genetics Institute) (Sarantou et al, 1997).

The automated Southern blot assay system provides RT-PCR product verification, with the highest stringency in blotting and sensitivity. Any discrepancies or faint signals in Southern blotting were repeated for verification. In each experiment set-up, samples of RT-PCR reagents without mRNA, donor PBL, and lymph node RNA from non-cancer patients were used as negative controls throughout RT-PCR procedures. Complementary DNA (cDNA) template for the cDNA probe and previously tested positive tumor lines or tumor biopsy specimens were included in the assay as positive RT-PCR controls. Southern blots were scanned on an electronic imager, recorded using computer software, and verified by at least two readers for positive and negative results. A positive result was recorded only after verification by Southern blotting.

RESULTS
Marker expression in breast cell lines and primary breast tumors
The 4 breast cancer cell lines assessed expressed by all five mRNA tumor markers (figure 2).Biopsies from 8 primary infiltrating ductal carcinomas (IDC) were evaluated for mRNA tumor marker expression. The primary breast tumors expressed all markers, except two tumors which did not express CK20.

Marker expression in normal PBL

PBL from 13 volunteer healthy donors were evaluated for mRNA marker expression . False-positive results were expressed in 10 of 13 (78%) PBL using CK-19, 6 of 13 (46%) PBL using CEA, 6 of 13 (46%) using MUC-1, and in 7 of 13 (54%) PBL using GA733-2 (Table 5). No false-positives were found in the normal PBL using CK-20. RT-PCR results were confirmed by EtBr and Southern blot assay.

TABLE 5
RT-PCR & SOUTHERN BLOT ANALYSIS OF MRNA MARKER EXPRESION

| Cell Line Marker | SPECIMEN | | |
	Breast Tumor (n=4)	Donor PBL (n=8)	Normal LN (n=13)
CEA	4/4 (100%)	8/8 (100%)	6/13 (46%)	3/3 (100%)
CK-19	4/4 (100%)	8/8 (100%)	10/13 (78%)	3/3 (100%)
CK-20	4/4 (100%)	6/8 (75%)	0/13 (0%)	2/3 (67%)
GA733.2	4/4 (100%)	8/8 (100%)	7/13 (54%)	3/3 (100%)
MUC-1	4/4 (100%)	8/8 (100%)	6/13 (46%)	3/3 (100%)

RT-PCR marker expression refers to RT-PCR and Southern blot positive. All specimens assessed were *B*-actin RT-PCR positive by EtBr analysis. ( ) refers to % positive. Annealing temperature of $60^{O}C$ used for CEA, CK-19, and CK-20. Annealing temperature of $55^{O}C$ used for GA733.2 and $55^{O}C$ for MUC-1.

Marker expression in normal lymph nodes

After informed consent normal lymph nodes (confirmed histopathologically), were obtained from patients undergoing non-cancer surgery. MUC-1, GA733-2, CEA, CK-19 and CK-20 were expressed in all of the normal lymph nodes tested by RT-PCR (Table 5).

CONCLUSIONS

The sensitivity and specificity of mRNA marker expression by RT-PCR must be assessed to determine the reliability of this ultra-sensitive molecular technique in comparison to conventional H&E and immunohistochemistry.

Most studies have relied on a single tumor marker to detect micometastases by RT-PCR even though tumors have been shown to be heterogeneous in gene expression. Tumor cells within a primary tumor may all express the same marker gene, but the level of specific mRNA can vary. In our study, none of the 5 mRNA markers were expressed in all 10 axillary lymph nodes containing metastases by H&E or IHC. In contrast, Mori et al (1995) reported CEA mRNA expression by RT-PCR in 30 of 30 lymph nodes containing metastases by histopathology. Similarly, Noguchi et al (1996) detected mRNA expression for both the MUC-1 and cytokeratin-19 genes by RT-PCR in all 10 axillary lymph nodes containing metastases. Schoenfeld et al (1994) detected cytokeratin-19 mRNA expression by RT-PCR in all 18 axillary lymph nodes with metastases. The 100% correlation of RT-PCR mRNA expression in axillary lymph nodes

containing metastases by conventional histopathology are probably related to the high sensitivity but low specificity of the assays used by other institutions.

Occult disease may go undetected in the axillary lymph node by RT-PCR because of a sampling error. Hematoxylin and eosin staining (H&E), IHC, and RT-PCR are all performed on different sections of the same lymph node. A lymph node with a small tumor burden (i.e. a few clusters of cells) located in the subcapsular sinus may not be included in the specimen assessed by RT-PCR. It is our policy to bi-valve the lymph node and take frozen sections from both sides. Even with this technique a sampling error may occur because the tumor cells may not be included in the section evaluated by RT-PCR. In addition to sampling error, the level of mRNA expression by the tumor cell may be too low to be amplified by RT-PCR or the tumor cell may not express the gene at all in early stages of tumor growth. A multiple mRNA RT-PCR assay, which takes into account the heterogeneity of tumors, may improve the sensitivity of detecting occult metastases.

The specificity of the RT-PCR and Southern blot assay is dependent on the normal lymph nodes not expressing the mRNA marker gene. In this study all five mRNA markers were expressed in normal lymph nodes by RT-PCR and confirmed by Southern blot analysis. Attempts to improve the specificity of the assay by increasing or decreasing the annealing temperature still resulted in the expression of these mRNA markers in normal lymph nodes (refer to table showing different annealing temperatures). Several institutions when using ethidium bromide staining instead of Southern blot analysis to evaluate RT-PCR products have not detected Cytokeratin-19, CEA, or MUC-1 expression in normal lymph nodes (Mori et al, 1995; Noguchi et al, 1996). Investigators argue that Southern blot is too sensitive and does not correlate with clinical outcome. However, ethidium bromide is very subjective in determining mRNA expression in contrast to Southern blot analysis. The cytokeratins, comprised of 20 different isotypes and predominantly expressed in epithelial cells, has been reported by several institutions as a target gene for detection of occult metastases by RT-PCR (Schoenfeld et al, 1994; Burchill et al, 1995). However, Burchill et al (1995) showed cytokeratin-19 to be expressed in 6 of 15 blood samples from healthy donors. Datta et al (1994) who related these false-positives to a pseudogene of cytokeratin-19, performed nested primer PCR to distinguish cytokeratin-19 gene from the pseudogene and did not detect false-positives in the blood of 10 healthy donors. In contrast, Schoenfeld et al (1994), performed nested primer PCR in axillary lymph nodes and detected false-positive expression of cytokeratin-19 in lymph nodes from patients without cancer. Schoenfeld (1994) recommended 40 cycles of PCR and Southern hybridization when using cytokeratin-19 as a marker. In our study, even when lowering the cycles of PCR to 35, cytokeratin-19 was found to be expressed in normal lymph nodes by both EtBr and Southern blot analysis (figure1). Cytokeratin-20 was also expressed in normal lymph nodes. The expression of these mRNA markers by RT-PCR in normal lymph nodes limits their use in improving detection of occult metastases compared to conventional H&E and IHC.

False-positive mRNA expression in normal lymph nodes by RT-PCR can result from a pseudogene being expressed as seen with cytokeratins. Designing primers to differentiate the pseudogene from the actual gene can prevent false-positive results. A quantitative value cannot be assigned to RT-PCR results that determines the level of mRNA expression. Consequently, a normal cell that expresses a particular gene at a very low level in comparison to a cancer cell will be considered a positive result by RT-PCR.

Lymph nodes taken from the axillary basin for RT-PCR analysis for the detection of occult metastases may require a higher stringency in comparison to blood to prevent false-positive results.

The detection of occult metastases in the axillary lymph nodes by RT-PCR is limited by the expression of mRNA markers in normal lymph nodes. Further investigation to identify mRNA markers limited in their expression in normal tissues or those expressed only in only tumor cells is needed to improve the sensitivity and specificity of the RT-PCR assay.

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FIGURE LEGEND

Figure 2. Representative examples of RT-PCR and Southern blot assay of breast cancer patients for the 5 mRNA markers. Lanes 1 & 2, breast cancer cell lines; lanes 3,4 & 5, breast tumor biopsies; and lane 6, positive marker Southern blot standard control.

Publications (to date)

1. Bostick P, T. Sarantou, K. Huynh, A. Conrad, A. Giuliano, D. Hoon. A multiple marker RT-PCR assay for detection of occult metastasis in frozen sections of sentinel nodes from breast cancer patients. Proc. Amer. Soc. Clin. Oncology 16: 153a, 1997. Presentation (Abstract)

2. Sarantou T., P. Bostick, D. Chi, A. Conrad, P. Schmid, A. Giuliano, D. Hoon. Detection of occult tumor cells by RT-PCR in frozen sections from breast sentinel lymh nodes. Soc. Surgical Oncology, 1997. Presentation (Abstract)

FIGURE 1

FIGURE 2